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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The hepatocyte growth factor (HGF)/Met signaling pathway has been shown to be important for stimulating cell proliferation, motility, invasion and metastasis. Recent work from our lab has identified a 60 kDa fragment from the carboxy-terminal domain of Met that localizes to the nucleus. Preliminary data from our also indicates that Met is translocated to the nucleus during <i>in vitro</i> wound healing of epithelial sheets of cells, but appears to be antibody dependent as a newly validated mouse monoclonal antibody does not detect the 60 kDa fragment. Because several pharmaceutical companies are currently developing Met-based therapies, it becomes even more important to gain an understanding of the role of nuclear Met, especially whether or not it may be contributing to invasion and metastasis. To date, no studies have been conducted to understand this aspect of Met function. Therefore the objectives of my proposal are to assess the role of Met in a model of epithelial-mesenchymal transition (EMT), identify key residues in the Met receptor necessary for nuclear translocation, and determine the functional role of Met in the nucleus.					
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**Introduction:**

The hepatocyte growth factor (HGF)/Met signaling pathway has been shown to be important for stimulating cell proliferation, motility, invasion and metastasis. Activating Met mutations are thought to contribute to the cause of several cancer types including papillary renal carcinoma, lung cancers, head and neck cancers and gastric cancer (1). Increases in Met expression have also been correlated with increased aggressiveness of cancer (2) and Met expression is both highest and strongly nuclear at the invasive front in breast carcinoma (3). Recent work from our lab has also demonstrated patients with high levels of nuclear Met have a worse prognosis even in lymph node negative breast cancer (4, 5) and has also identified a 60 kDa fragment from the carboxy-terminal domain of Met that localizes to the nucleus. Preliminary data from our lab also indicates that Met is translocated to the nucleus during *in vitro* wound healing of epithelial sheets of cells. Because of this, the underlying hypothesis of this proposal is: the translocation of Met to the nucleus leads to interactions and functions contributing to cancer progression by promoting epithelial-mesenchymal transition. Because several pharmaceutical companies are currently developing Met-based therapies, it becomes even more important to gain an understanding of the role of nuclear Met, especially whether or not it may be contributing to invasion and metastasis. To date, no studies have been conducted to understand this aspect of Met function.

**Body:**

The goal of this research is to assess the role of the Met receptor in a model of epithelial-mesenchymal transition (EMT) in addition to identifying key residues of the Met receptor necessary for nuclear translocation and to determine the functional role of Met in the nucleus.

The original statement of work was the following:

Task 1: Identification of genes regulated by nuclear Met

- a. Perform ChIP on MCF7 breast cancer and HMEC normal breast cell lines under normal epithelial conditions and after triggering of a mesenchymal transition
- b. Send isolated DNA fragments to the 454 company for sequencing
- c. Align sequencing results and determine frequency distributions for promoter regions

Timeline: Months 1-6

Task 2: Validation of ChIP-454 promoter sequences

- a. Clone promoter sequences into luciferase reporter plasmids
- b. Transfect into cell lines with strong nuclear Met and measure luciferase expression
- c. Transfect into cell lines with membranous Met inhibited with PHA and measure luciferase expression

Timeline: Months 5-12

Task 3: Determine the role of Met in epithelial-mesenchymal transitions

- a. Use siRNA to knock down Met expression in LIM1863 cells and a scrambled RNA as a control for specificity and/or treat the cells with PHA to inhibit Met kinase activity and nuclear translocation
- b. Confirm specific inhibition of Met expression and/or activity by western blot
- c. Treat LIM1863 organoids with recombinant TGF $\beta$  and TNF $\alpha$  in the presence of Met inhibition compared to control treated with scrambled RNA or not inhibited with PHA

- d. Verify EMT in control cells by loss of E-cadherin and migration of cells from the organoid to form a monolayer and use these markers to determine if Met inhibition prevents this transition

Timeline: Months 11-24

Task 4: Generate stable MCF7 and CaCo2 cell lines expressing tagged Met

- a. Clone full-length Met-GFP and Met-HA epitope tag into retroviral vectors
- b. Transfect into HEK293T cells to obtain packaged viral particles and use to generate MCF7 and CaCo2 stable cell lines expressing these constructs
- c. Perform a wound healing assay with each of the stable lines and determine subcellular localization of full-length Met-GFP or Met-HA

Timeline: Months 23-30

Task 5: Identify residues necessary for nuclear translocation of Met

- a. Use site-directed mutagenesis on the full-length Met construct to create a deletion series of the juxtamembrane domain and full-length constructs with alanine substitutions at residues Y1349, Y1356, or both Y1349 and Y1356
- b. Transfect into HEK293T cells to obtain packaged viral particles and use to generate MCF7 and CaCo2 stable cell line clones expressing each of the above constructs
- c. Perform a wound healing assay with each of the stable lines and determine the subcellular localization of each mutant construct

Timeline: Months 26-36

The original goal of Task 1 was to identify genes regulated by nuclear Met through 454-sequencing of DNA isolated by ChIP of wounded versus un-wounded cells. As reported last year, the 454 company was unable to amplify from the isolated DNA we originally sent. This led to our realization that the manner we were using to generate nuclear Met (wounding of the cellular monolayer) would not generate uniform transcriptional changes in all of the cells as we believed only cells at the wound edge expressed nuclear Met (Figure 3). In addition, a number of the transcriptional changes we may have identified would have been as a result of the wound healing process and not necessarily directly related to the presence of nuclear Met, decreasing the chance they would be verified as Met targets in Task 2. To increase the likelihood that we would successfully identify genes modulated by nuclear Met, my initial efforts focused on Task 4: generation of stable cell lines expressing Met tagged with GFP. Having cell lines stably expressing either the full-length Met-GFP, the cytoplasmic domain of Met known to be necessary for nuclear localization, and a truncated c-terminal domain of Met previously shown to by our lab to be excluded from the nucleus (6) would allow us to compare transcriptional profiles of MCF7 lines either expressing nuclear Met or Met excluded from the nucleus. First attempts to generate stable lines were made using the constructs previously generated in the lab (6) for transient transfections. MCF7 cells were transfected with Lipofectimine and grown in selection media with G418 theoretically selecting for cells maintaining expression of Met-GFP. Generation of MCF7 cells stably expressing the GFP control was successful, but all attempts at obtaining stable expression of Met-GFP using this method were not successful. At the time of last year's report we had just begun to have success in cloning the Met-GFP constructs into lentiviral/retroviral vectors. In the past year we were successful in cloning both the full-length Met-GFP and the CytoMet-GFP constructs into the pBABE retroviral vector containing a

puromycin selection marker. Total cell lysates from pools of puromycin resistant HEK293T cells showed that the cells were expressing low levels of CytoMet-GFP, however the full-length Met-GFP was not detected (Figure 13). Furthermore we determined that the full-length Met-GFP sequence was integrated into the genomic DNA of 3 different cell lines that we had infected, HEK293T, MDA-MB-231, and NIH3T3 (Figure 14) even though no expression could be detected at the protein level. Even though the CytoMet-GFP did appear to be expressed, we were unable to expand clones of expressing cells as the tagged protein expression was lost within a passage of the cells infected. Similar results of undetectable protein expression were obtained when transiently transfecting cells with a full-length Met with a FLAG-6His tag, indicating that the nature of the tag does not seem to be the issue (data not shown). As a last attempt at generating cells expressing Met-GFP we are in the process of cloning into an inducible vector system.

Task 3 was to determine the role of Met in epithelial-mesenchymal transitions. Before moving to a 3-dimensional model system we have focused first on analyzing the role of Met in wound healing of cellular monolayers. Much of the work in my previous report focused on the development of our wound healing assay (Figure 1). We have also demonstrated that as expected, HGF treatment (50 ng/ml) and therefore Met activation increases the healing response or Met inhibition with the Pfizer compound PHA-66552 prevents wound healing. Phase contrast examples of this are shown in Figure 2 for H1650 cells and overall healing is quantified in Figure 3. Similarly, BT20, A431, and CaCo2 cell lines also demonstrate the same patterns when quantified and results for all cell lines are shown in Figure 3. In addition to the overall wound healing response dependency on Met expression, immunofluorescence staining of H1650 cells shows that Met is localized to the nucleus at the wound edge when stained with a polyclonal c-terminal specific antibody for Met. 60x deconvolution images are shown in Figure 4 for H1650 cells treated with HGF and stained 8 hours after wounding with CVD13, a c-terminal Met Ab. Interestingly, immunofluorescence staining of H1650 cells 8 hours after wounding also shows that nuclear Met is also inhibited by PHA treatment, even in the presence of HGF (Figure 5a). Since my last report I have also quantified the percentage of nuclei Met positive and determined that HGF does appear to increase the number of nuclei positive whereas either Pfizer inhibitor PHA or PF-1066 decreases the overall percentage of nuclei Met positive (Figure 5b). We have also observed nuclear Met at the wound edge in a number of other cell lines and an example in A431 cells is shown in Figure 6.

We next sought to determine if nuclear localization of Met was a function of either proliferation or migration of the cells. To address this, H1650 cells were pretreated with either Rockout (50  $\mu$ M) to inhibit migration or Mitomycin C (10  $\mu$ g/ml) to inhibit proliferation and then stained with CVD13 to assess Met localization (Figure 7a). The percent nuclei positive were also quantified (Figure 7b) and indicated that nuclear localization was not dependent on either proliferation or migration alone. Along with these results we were also concerned if the nuclear localization we were observing was truly a function of the wounding of the cells and sought to better standardize the plating density and timing of these studies. To this end a time course was conducted with H1650 cells to determine how many days post plating the cells the Met localization was membranous. Unfortunately, our results were that cells plated at identical densities for identical times would result in differences in Met localization as assessed by CVD13 staining (Figure 8). This was very concerning and led us to search for a cell line where

we could consistently see membranous Met localization to allow us to probe for what triggers Met nuclear localization. We observed that A431 cells were generally membranous regardless of the time point when stained with CVD13. We also wanted to confirm that the staining we were seeing was specific to Met and not a potentially non-specific interaction of using a polyclonal antibody. We obtained a mouse monoclonal, c-terminal specific antibody from George Vande Woude and validated its specificity using our H1650 and CaCo2 cell lines stably expressing Met shRNA (Figures 9 & 10). From this point forward, the goal of our work has been to confirm the nuclear localization of Met with this well-validated monoclonal antibody. The most compelling data we sought to reproduce with this antibody was the presence of a ~60 kDa fragment in nuclear fractions from either H1650 or A431 cells. Unfortunately, we were not able to reproduce this fragment in either cell line using our previously published sucrose gradient fractionation method (6) or the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) (Figures 11 and 12). This could be because the 60 kDa band is not recognized by the mouse monoclonal antibody or this fragment we believed to be Met-specific could in fact be a Met-related protein causing cross-reactivity with the polyclonal Met antibodies.

In parallel we sought to examine if loss of E-cadherin in A431 cells would lead to nuclear localization of Met. E-cadherin was inhibited with DECMA-1 treatment (24 µg/ml) for 48 hours and the Met staining pattern became more diffuse, but did not appear to be nuclear (Figure 15). A431 cells were also infected with a lentiviral plasmid containing an E-cadherin shRNA, which reduced the E-cadherin expression levels ~50%, however the Met localization remained diffuse cytoplasmic, similar to that seen with the DECMA-1 inhibition. The lack of observed nuclear localization could again be due to the monoclonal antibody not being able to recognize the smaller Met fragments.

No work has been completed on Task 5 to date. Completion of viral infection of MCF7 cells with Met-GFP to generate stable cell lines will be necessary before this task can be addressed and due to the issues in generating Met-GFP stable lines may fall outside the timeline of this proposal.

#### **Key Research Accomplishments:**

- Generation of pBABE-Met-GFP and CytoMet-GFP
- Reproducible method for wound healing and quantification
- Nuclear Met is observed at the leading edge of the wounds
- Nuclear Met is inhibited in H1650 cells when treated with a small molecule inhibitor
- Nuclear Met is phosphorylated in H1650 cells
- Validation of c-terminal specific mouse monoclonal Met antibody
- Met19s (monoclonal) antibody does not recognize 60 kDa Met
- Inhibition of E-cadherin by DECMA-1 or shRNA does not lead to nuclear Met localization as determined with Met19s

#### **Reportable Outcomes:**

Presentation: Yale University Department of Pathology Research in Progress

Poster: Met (the HGF receptor) localizes to the nucleus in wound healing.

AACR, April 2010, Washington, D.C.

### **Conclusion:**

In conclusion, my results to date demonstrate nuclear Met is present in the wound edge and is not inhibited by the inhibition of either migration or proliferation as determined when Met is stained with CVD13 (polyclonal). The inconsistent results we obtained when plating H1650 cells at uniform densities led us to question if the antibody and cell line used was appropriate. To address this we validated the monoclonal Met19s obtained from George Vande Woude, however we were not able to detect a 60 kDa fragment in fractionations using this antibody. Inhibition of E-cadherin by either DECMA-1 or shRNA did not cause the translocation of Met to the nucleus in A431 cells as determined by immunofluorescent staining with Met19s. The generation of MCF7 cells stably expressing Met-GFP will allow us to track the localization of Met in live cells after wounding and generation of cell lines expressing the predominately nuclear or cytoplasmic Met constructs will allow us to determine what the functional role of nuclear Met is, however progress has been hindered by the lack of Met-GFP expression at the protein level. We hope that generating an inducible Met-GFP will address these issues and allow us to confirm the nuclear localization of Met through live cell imaging. Determining the function of nuclear Met is extremely important as a number of pharmaceutical companies are currently in the process of developing cancer therapies based on inhibition of the Met receptor. Additional work from our lab has published that, in a cohort of 640 cases of invasive breast cancer on a tissue microarray, high levels of nuclear Met were associated with poor survival (4) and we were able to reproduce this result using Met19s on our breast cohort. This highlights the functional relevance of nuclear Met and understanding the mechanism and function behind nuclear Met could provide additional insight as to how this receptor functions in cancer progression.

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4. Pozner-Moulis S, Cregger M, Camp RL, Rimm DL. Antibody validation by quantitative analysis of protein expression using expression of Met in breast cancer as a model. *Lab Invest* 2007; 87: 251-60.
5. Camp RL, Rimm EB, Rimm DL. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. *Cancer* 1999; 86: 2259-65.
6. Pozner-Moulis S, Pappas DJ, Rimm DL. Met, the hepatocyte growth factor receptor, localizes to the nucleus in cells at low density. *Cancer Research* 2006; 66: 7976-82.



## Appendices & Supporting Data:

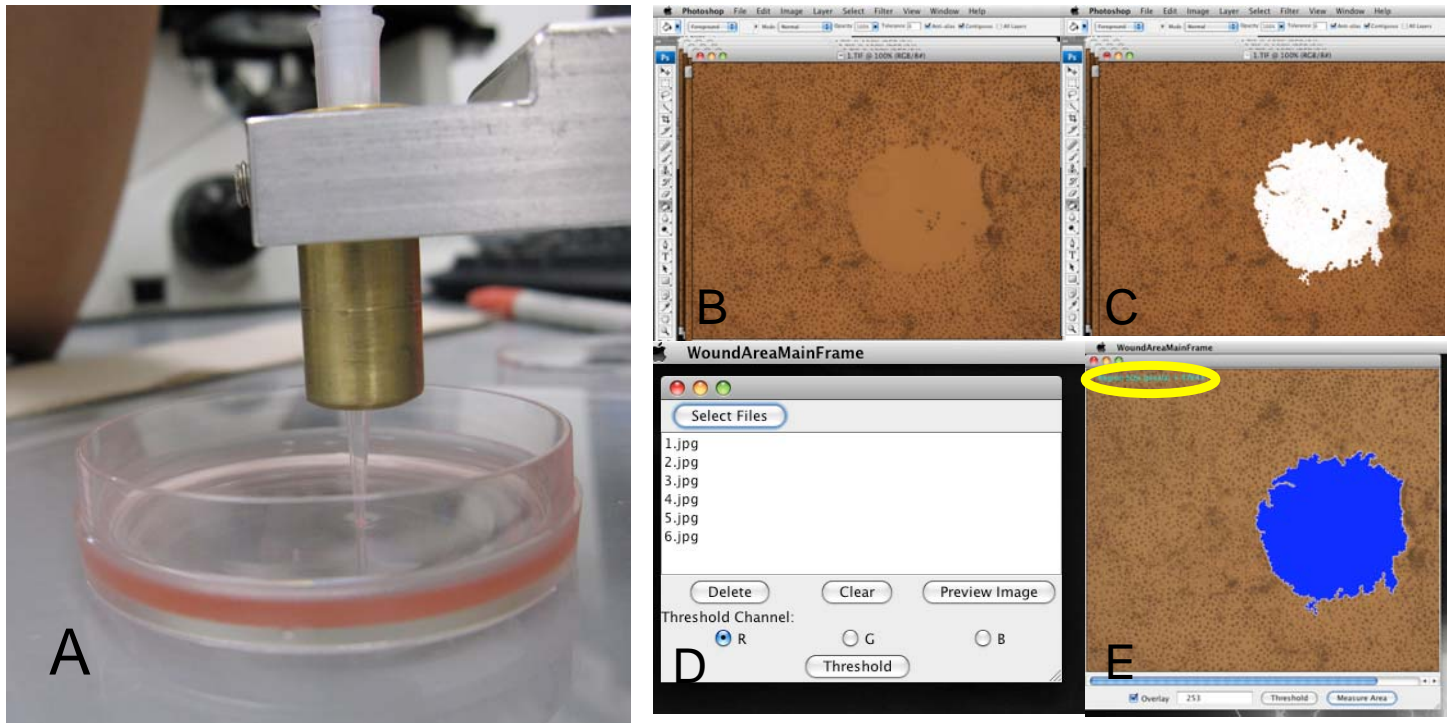


Figure 1: Wound healing method. A: 20 µl pipet tip is used to create circular wounds on a coverslip of confluent cells. B: Phase contrast images are taken of wounds at time 0 and final time point for each experiment. C: Wound area is filled with white using photoshop to allow for easy thresholding. D: WoundArea java program is used to select experiment files containing wound images. E: Image is thresh-holded to select the wound area and the total area (pixels) is displayed in the top left of the window (circled in yellow).

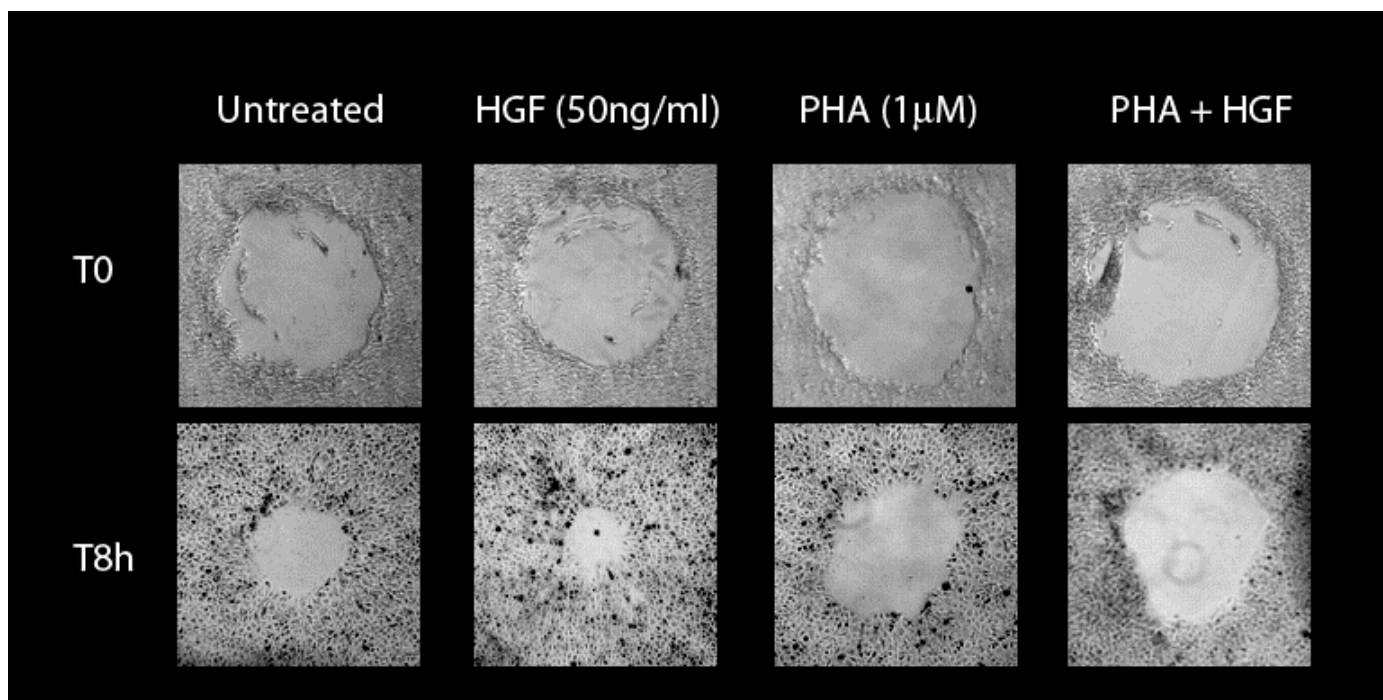


Figure 2. Wound healing of H1650 cells is increased upon HGF treatment and inhibited upon pretreatment of cells with PHA for 2 hours prior to wounding. Phase contrast images are shown of Time 0 (Top row) and Time 8 hours (Bottom row) for each condition: Untreated, HGF (50 ng/ml), PHA (1 $\mu$ M), and PHA (1 $\mu$ M) with HGF (50ng/ml) addition at time of wounding.

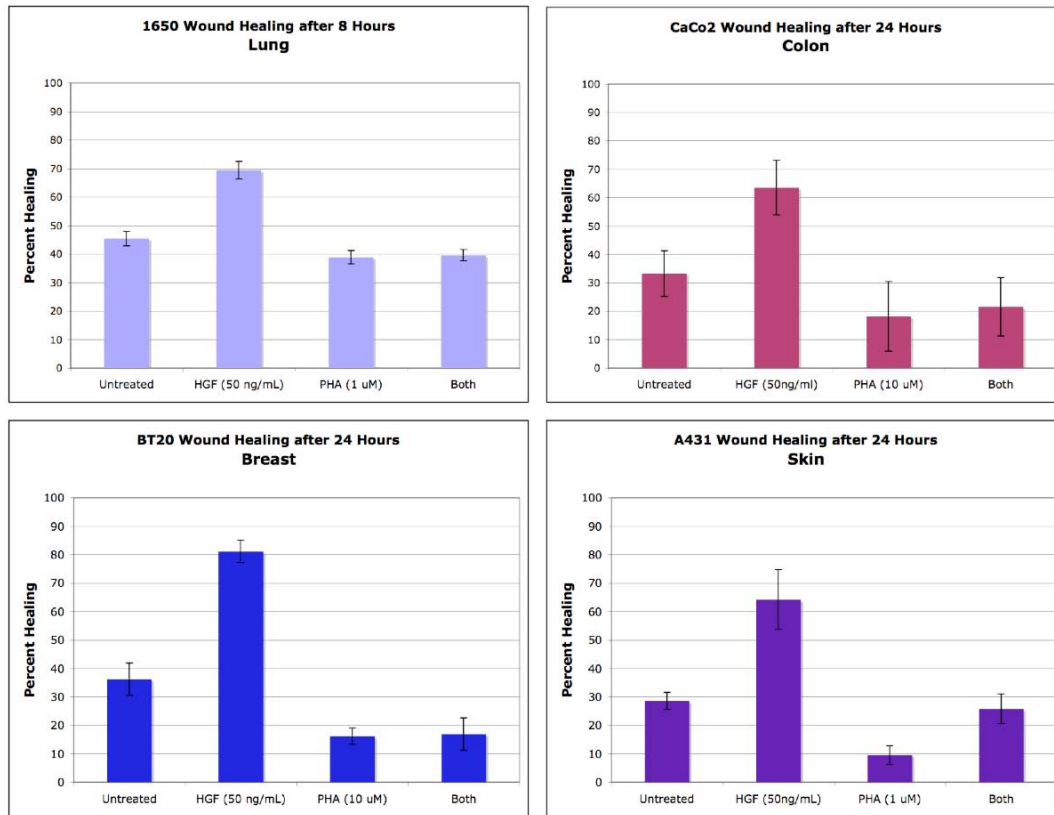


Figure 3. Total wound healing response in H1650, CaCo2, BT20, and A431 cell lines. Error bars represent standard error of the mean.

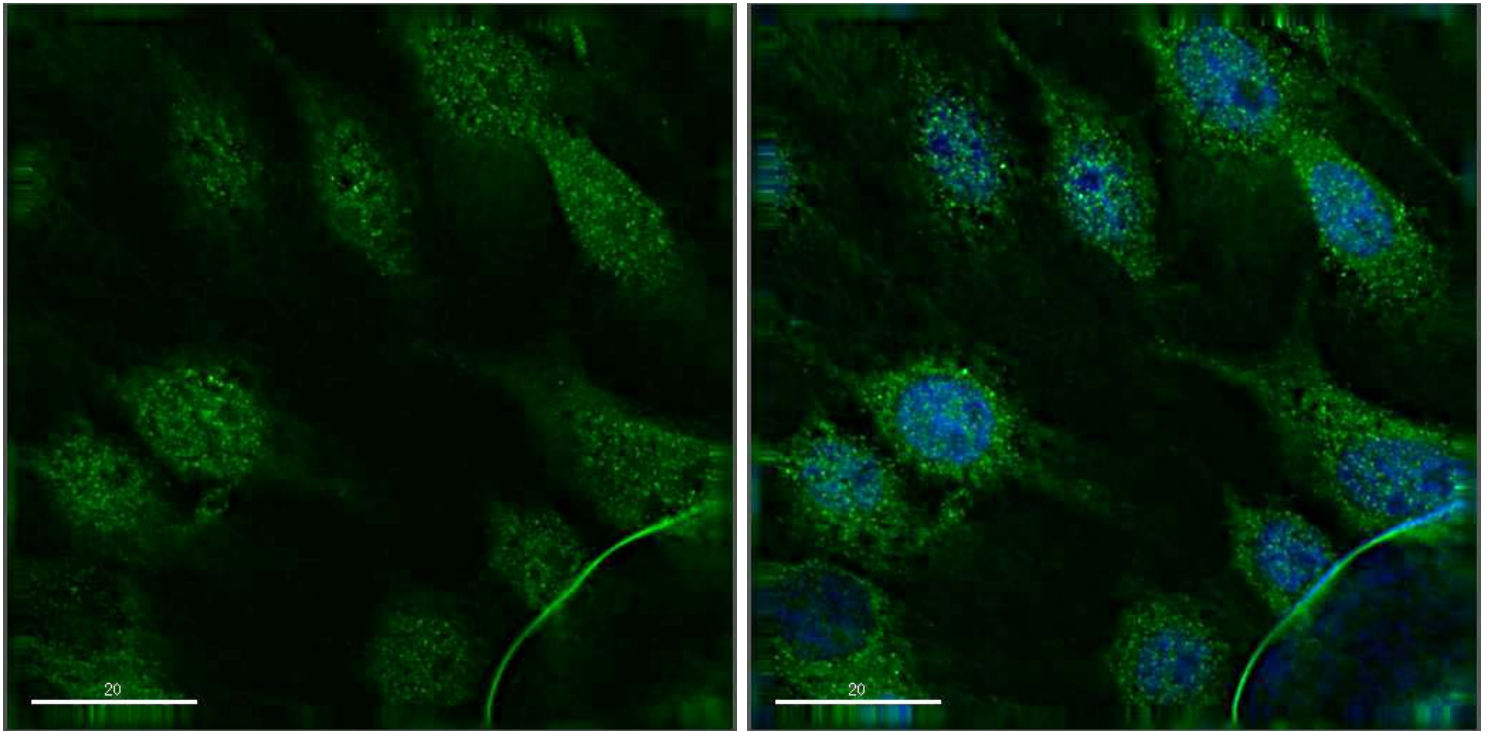


Figure 4. H1650 cells have nuclear Met at the wound edge after HGF treatment 8 hours post wounding. Green – stained for Met (CVD13 1:500) Blue – represents DAPI staining. Images are taken at 60x using a deconvolution microscope. Left: Met staining alone. Right: Met staining with DAPI overlay.

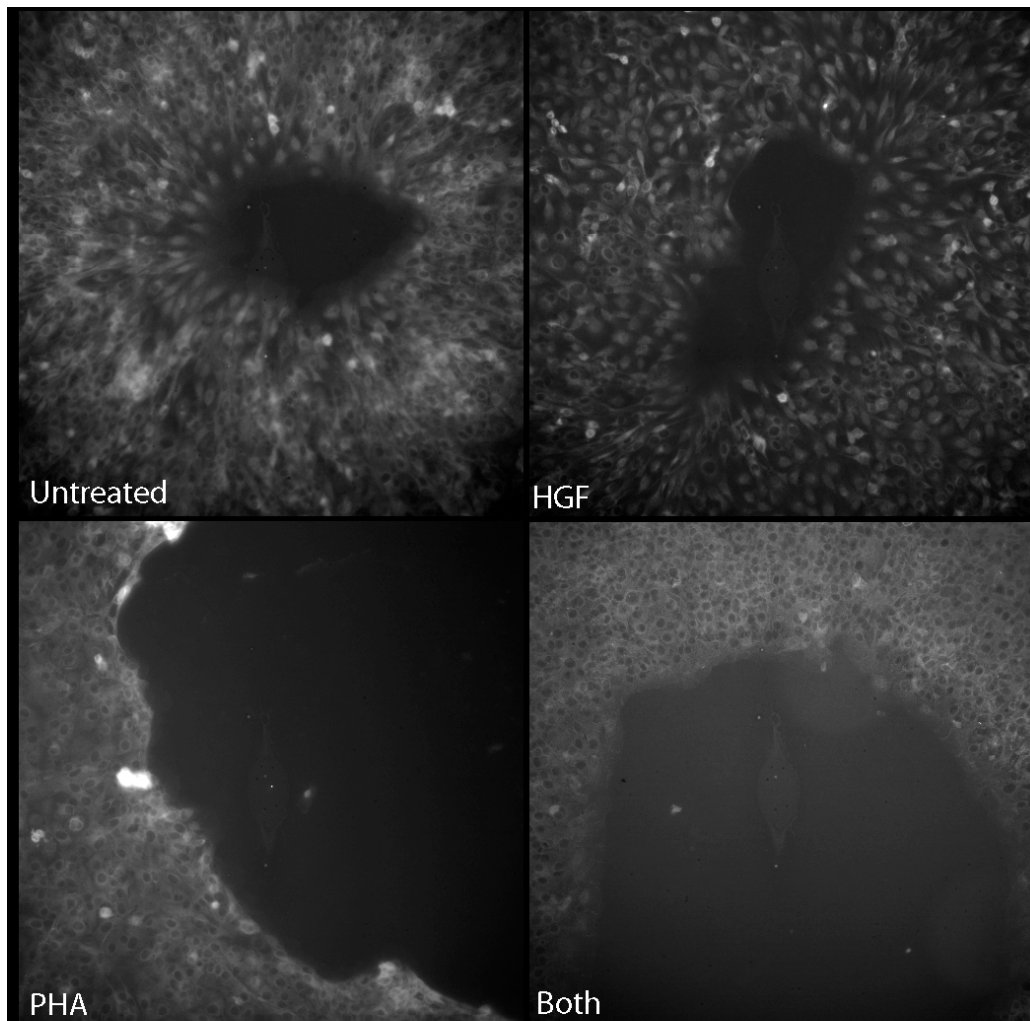


Figure 5a. Nuclear Met is present in the invasive front of wounded H1650 cells untreated and treated with HGF (50ng/ml) for 8 hours. Top left: Untreated cells. Top right, HGF stimulation. Bottom left, PHA inhibition 2 hours prior to wounding (1mM). Bottom right, PHA treatment 2 hours prior to addition of HGF and wounding. Cells were stained with Met Ab CVD13 (Invitrogen) 1:500.



Figure 5b. Nuclei positive for nuclear Met adjacent to the wound edge as assessed by immunofluorescence staining with CVD13 were counted and divided by the total number of nuclei adjacent to the wound edge as determined by DAPI staining to determine the percentage of nuclei Met positive in H1650 cells when stimulated with HGF alone or in the presence of either Pfizer Met inhibitor PHA or PF-1066.

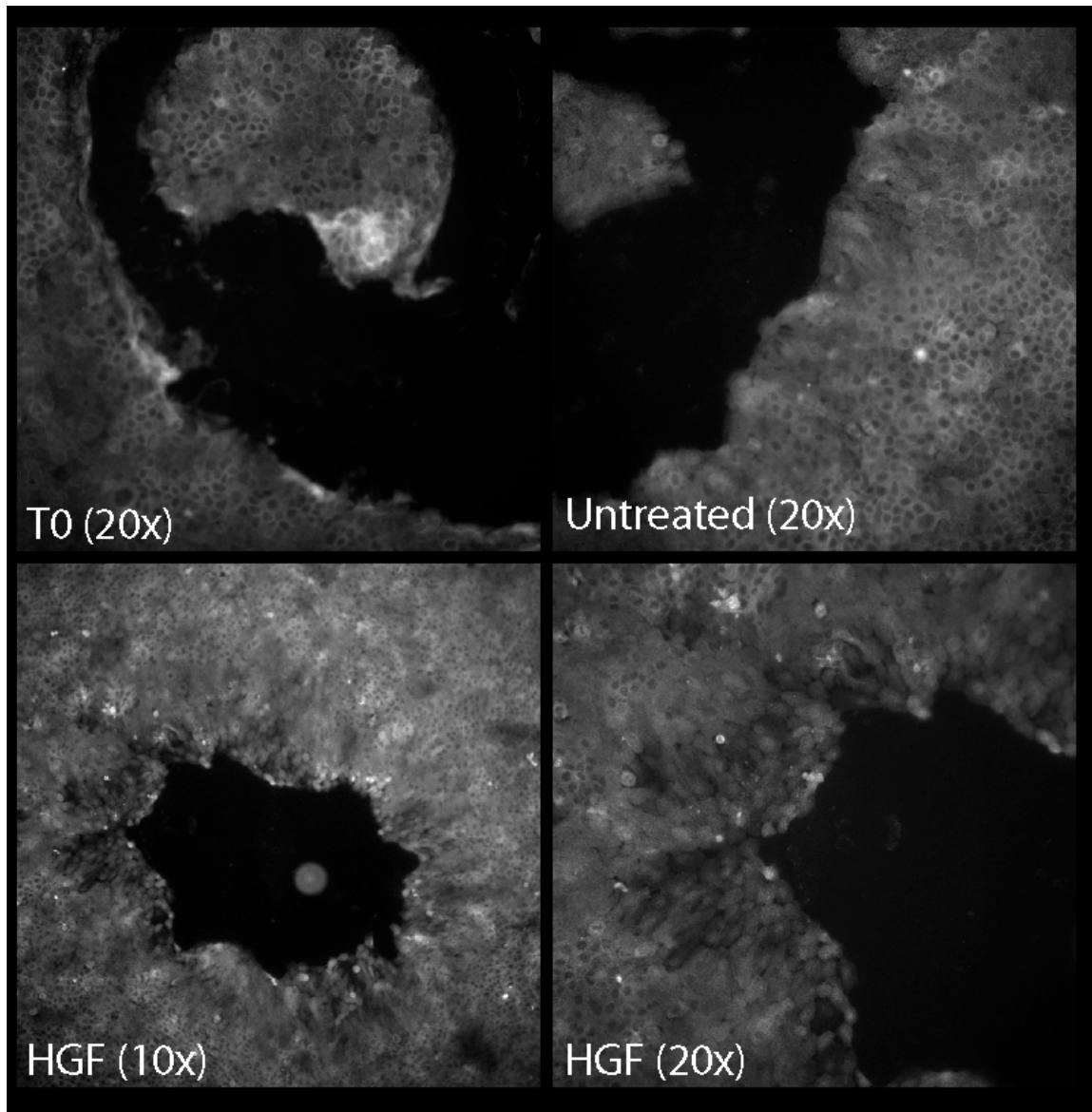


Figure 6. Nuclear Met is seen at the wound edge in A431 cells untreated and treated with HGF (50 ng/ml) after 24 hours. Top left: Untreated cells at time 0. Top right, Untreated cells 24 hours after wounding. Bottom left, HGF treatment 10x. Bottom right, HGF treatment 20x. Cells were stained with Met Ab CVD13 (Invitrogen) 1:500.



H1650 - 7 Hours Post Wounding

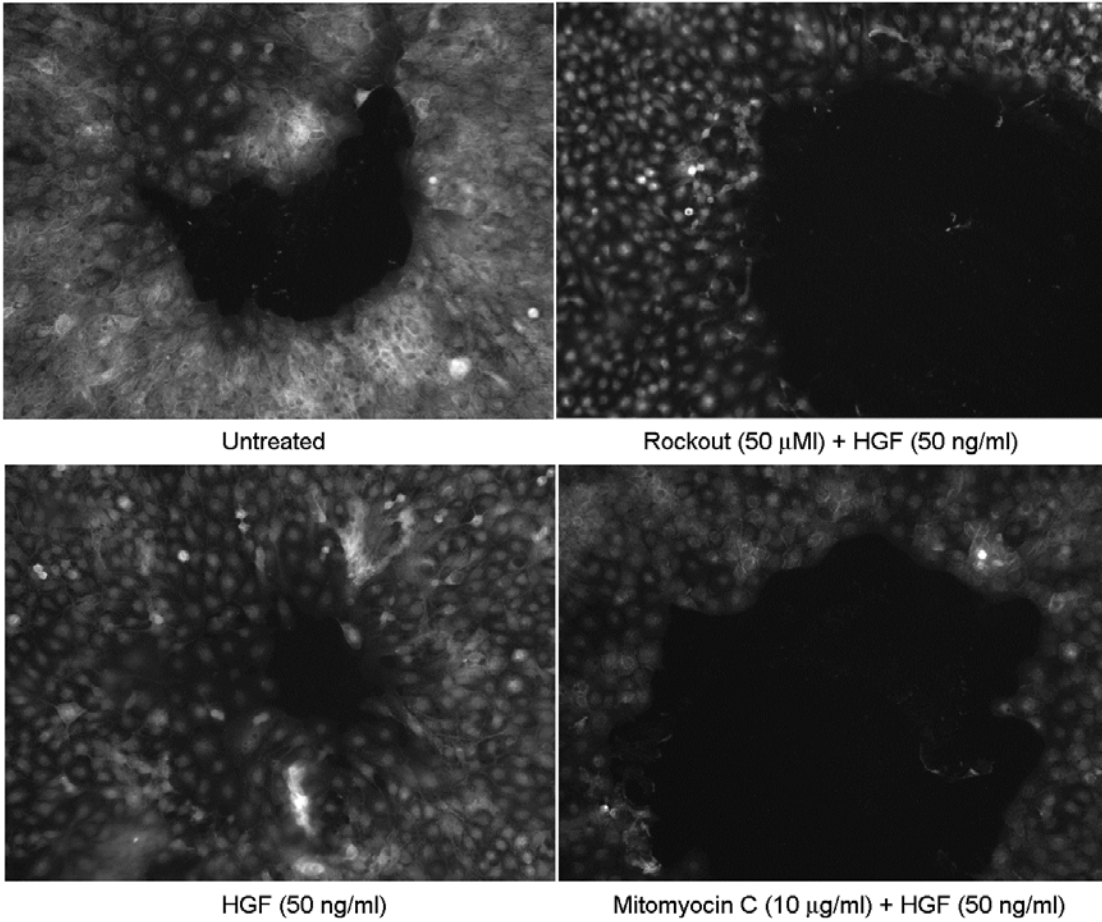


Figure 7a. H1650 cells were pretreated with either the cell migration inhibitor Rockout or the proliferation inhibitor Mitomycin C for 2 hours then HGF was added and the cells were wounded. The coverslips were fixed 7 hours post wounding and stained with CVD13 to assess Met localization. Nuclear Met does not appear to be dependent on either migration or proliferation alone.



Figure 7b. Nuclei positive for nuclear Met adjacent to the wound edge as assessed by immunofluorescence staining with CVD13 were counted and divided by the total number of nuclei adjacent to the wound edge as determined by DAPI staining to determine the percentage of nuclei Met positive in H1650 cells when stimulated with HGF alone or in the presence of either Mitomycin C or Rockout.

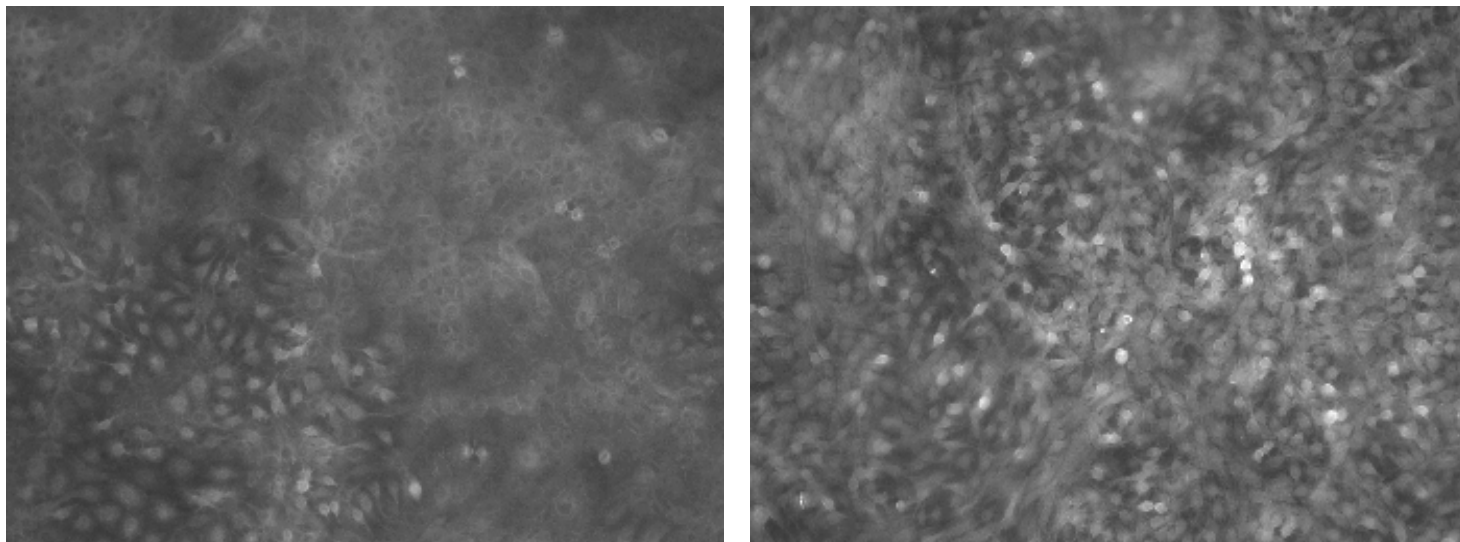


Figure 8. H1650 Plated at identical densities and grown for identical length of time resulted in different Met localization patterns when stained with CVD13, either between coverslips (left versus right) or between cells on the same coverslip (left).

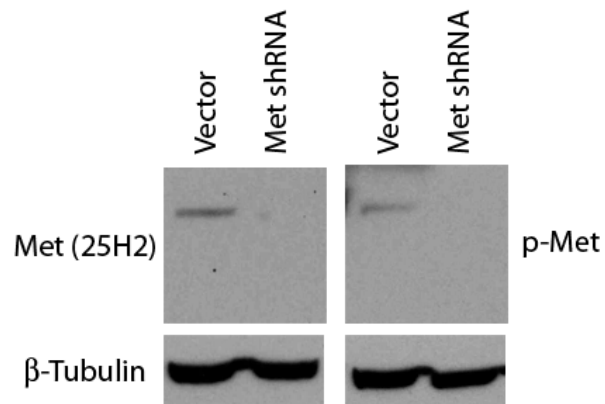
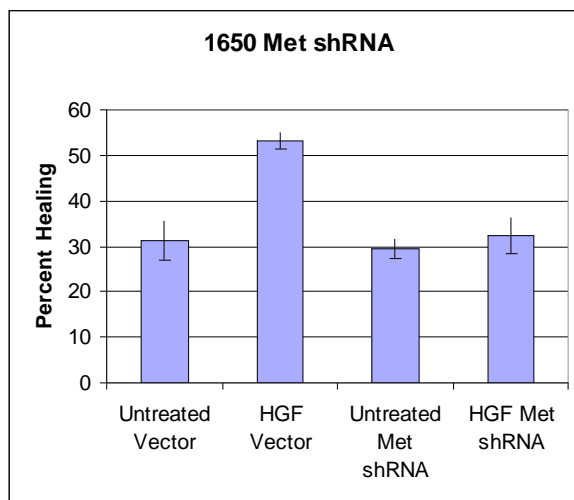


Figure 9. Left: Total wound healing response of H1650 control cells vs. Met shRNA knockdown cells untreated or stimulated with HGF. HGF stimulated healing response is abolished in knockdown cells. Right: WB of cell lysates shows efficacy of Met knockdown. Beta-tubulin is the loading control.

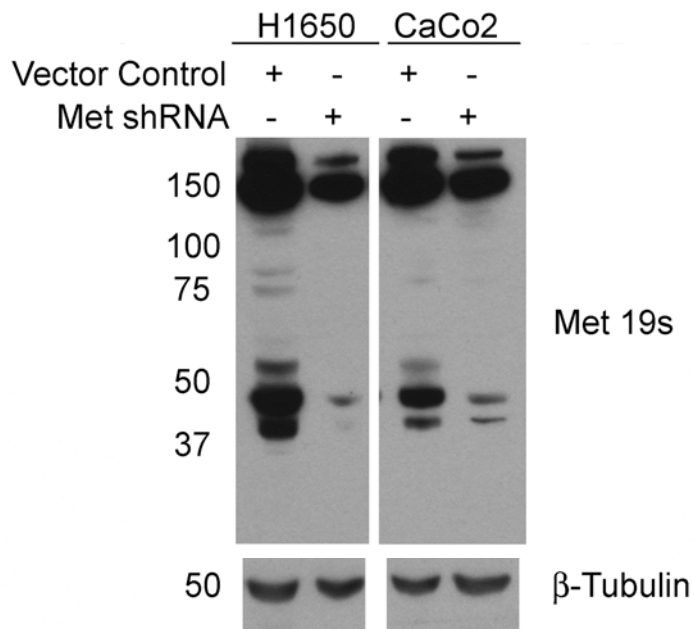


Figure 10. Met monoclonal antibody (Met19s) is specific. Whole cell lysates of H1650 and CaCo2 cells either infected with the vector control or the Met shRNA were blotted with Met19s (1:1000). All bands identified in the vector control were correspondingly reduced in the lysates of the knockdown cell lines.

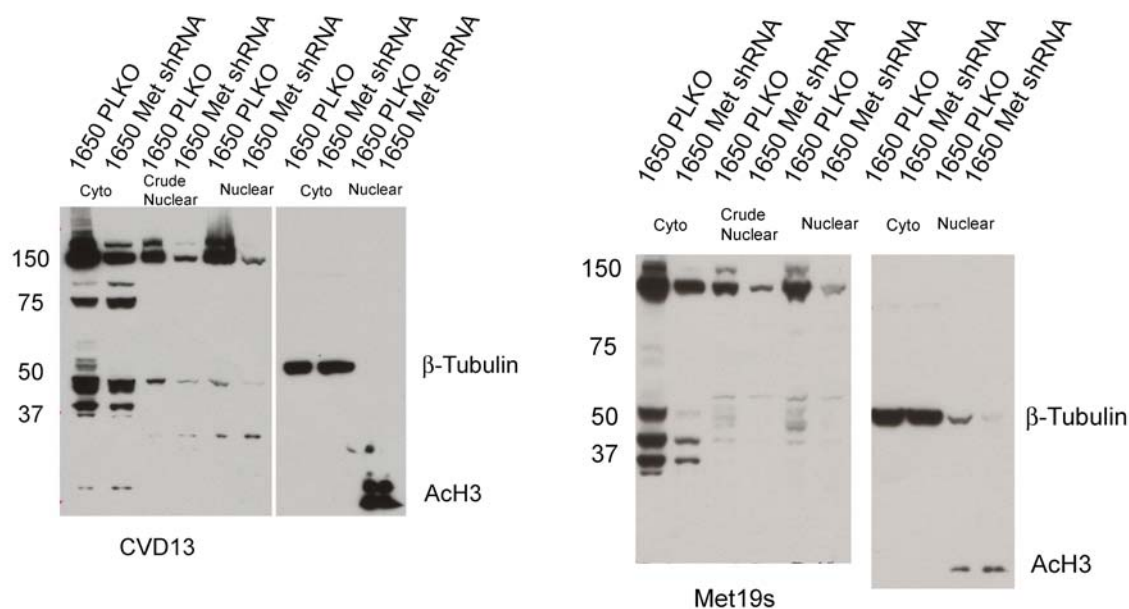


Figure 11. H1650 cells, either the PLKO vector control, or the Met shRNA infected lines were fractionated in differential sucrose buffers according to our published protocol (6) and blotted for either CVD13 or Met19s. Note the full-length pro-Met contamination in the nuclear fractions although tubulin and Ac-histone3 are clean. Also note the lack of any enrichment of a ~60 kDa fragment in the nuclear fraction.

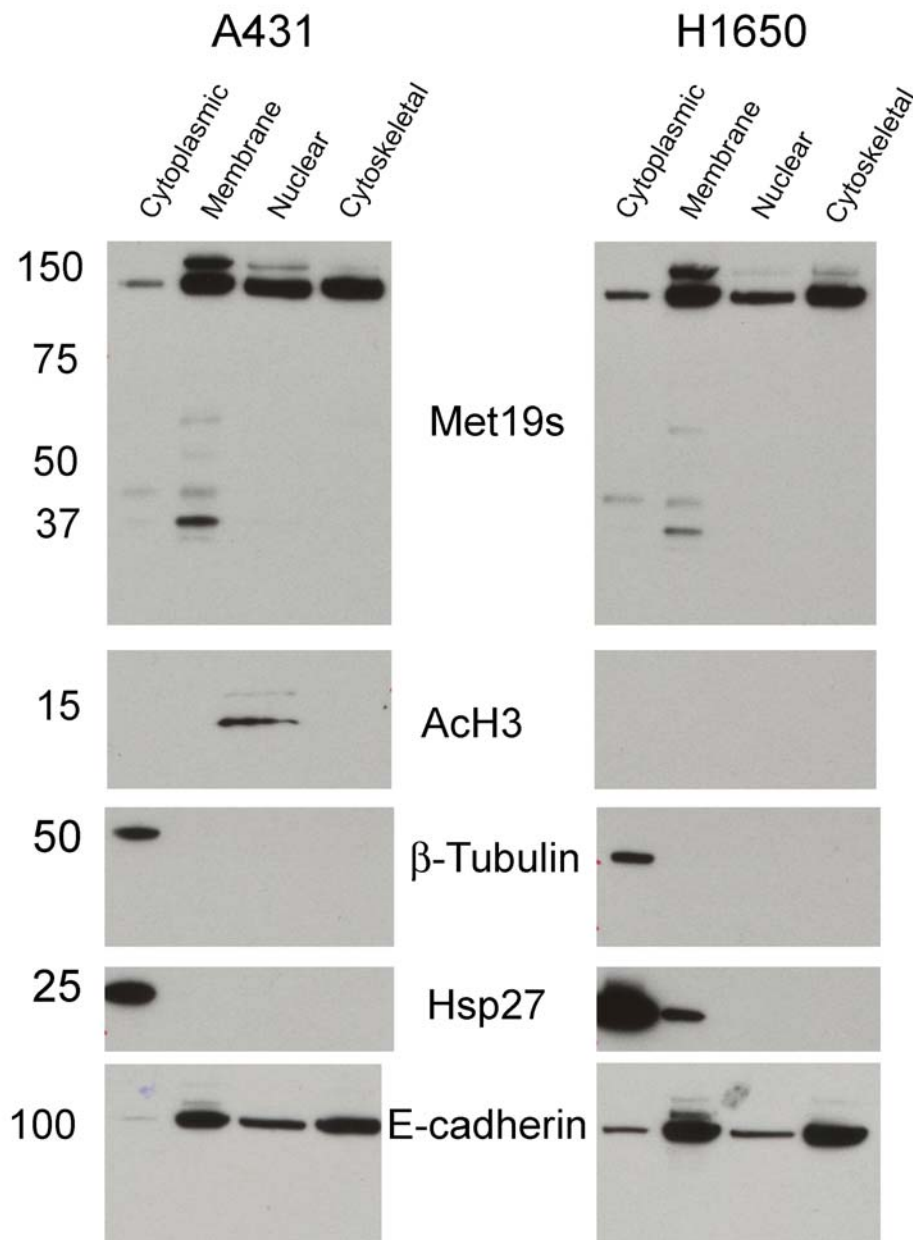


Figure 12. A431 and H1650 cells were fractionated using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to manufacturer's protocol. In both cell lines the only Met specific band recognized by Met19s monoclonal antibody in the nuclear fraction was for the full-length receptor. Smaller Met fragments were seen in the membrane fraction only. Ac-histone3 was only present in the nuclear fraction of the A431 cells and tubulin served as a control for the cytoplasmic fraction. Hsp27 was used to mark cytoplasmic and ER compartments. E-cadherin was used to determine if the full-length Met seen in the nuclear fraction was due to other membrane contamination.

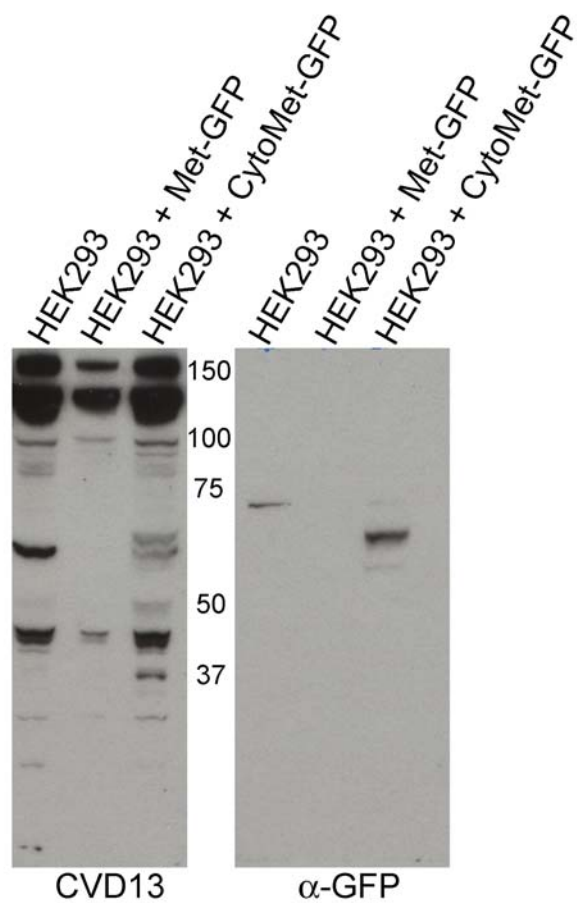


Figure 13. Whole cell lysates from HEK 293T cells infected with either Met-GFP or CytoMet-GFP were probed for total Met expression (Left) or GFP expression (Right). Only the Cyto-Met-GFP appeared to be expressed.

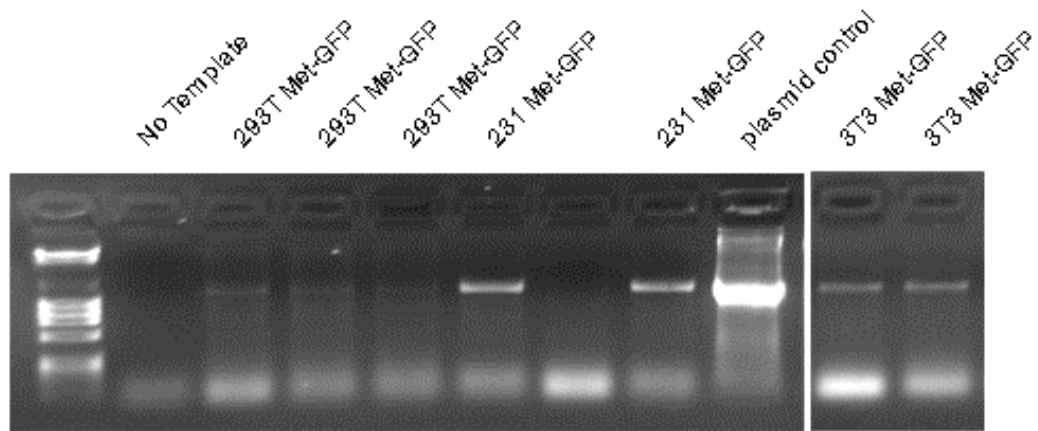


Figure 14. Genomic DNA was isolated from HEK293T, MDA-MB-231, and NIH3T3 cells that had been infected with our Met-GFP pBABE construct and PCR primers were used to detect only the presence of Met tagged with GFP. All cell lines tested were positive for a band the same size as the plasmid positive control.



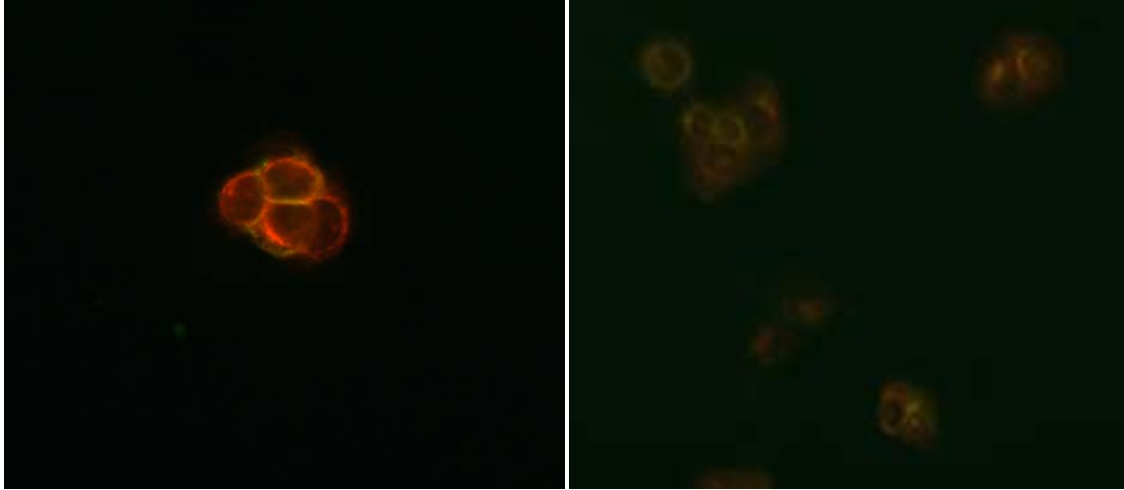


Figure 15. A431 cells grown in chamber slides for 48 hours either in normal serum (left) or with DECMA-1 (24 µg/ml) (right). Cells were fixed and double stained for Met (Met19s in red) and E-cadherin (CST rabbit monoclonal in green). Co-localization of Met and E-cadherin appears yellow. E-cadherin expression is reduced and Met localization becomes more diffuse cytoplasmic after DECMA-1 treatment.